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(54) Immunoassay of non-A, non-B hepatitis virus-related antigens, monoclonal antibodies for use therein, and hybridomas producing the antibodies

(57) This invention concerns a monoclonal antibody having binding specificity for an antigenic determinant site on core structural protein from Non-A,Non-B hepatitis virus (NANBV); a hybridoma cell line capable of producing the monoclonal antibody; a process for the preparation of the monoclonal antibody; an immunoassay of NANBV-related antigens by use of the monoclonal antibody; and a test kit for use in the immunoassay. The preferred monoclonal antibody is 5E3, 5F11, 515S or 1080S. The monoclonal antibody can specifically recognize the NANBV core structural protein in sera from patients with Non-A,Non-B hepatitis thereby being served extensively as an antibody in various immunological reagents for definitive diagnosis of Non-A, Non-B hepatitis.

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cured by the INF treatment alone, as seen from the fact that there are many cases re-suffering from the hepatitis even when the IFN treatment was terminated. Thus complete exclusion of NANBV from the patient bodies is difficult. It is rather significant to grasp the pathology of the disease in order to assume the treatment or prognosis, and because of this, it is strongly desired not only to detect antibodies to NANBV-related antigens but also to measure NANBV-related markers (i.e., antigens). Thus, the development of a method of obtaining NANBV virions, particularly NANBV-related antigens from infected sera in a simple way and in a high yield, as well as the detection or quantitative analysis of the virions or antigens, has been demanded.

Accordingly, an object of this invention is to provide a method for the simple treatment of NANBV-related antigens to concentrate and then denature them in specimens.

Another object of this invention is to provide a monoclonal antibody which can specifically recognize an NANBV-related core antigen and has a binding constant K_A of 5×10^7 [M^{-1}] or larger when forming an immune complex between the monoclonal antibody and the NANBV-related core antigen; a hybridoma capable of producing the monoclonal antibody; and a method for the preparation of the monoclonal antibody.

Yet another object of the invention is to provide a method for sensitively detecting or quantifying an NANBV-related antigen using the monoclonal antibody.

Embodiments of the invention are described below, by way of example only, and with reference to the accompanying drawings of which:

Figure 1 is a photograph showing Western blot images as results of the immunological reaction between each of the monoclonal antibodies 515S, 1080S, 5E3 and 5F11 and an HCV.CORE antigen, where
lane 1: lysate of a rabbit kidney culture cell line (RK13) infected with a recombinant vaccinia virus LO-R6J13, approximately 2.5×10^4 cells/lane, non-reducing;
lane 2: lysate of a rabbit kidney culture cell line (RK13) infected with the recombinant vaccinia virus LO-R6J13, approximately 2.5×10^4 cells/lane, reducing;
lane 3: lysate of a rabbit kidney culture cell line (RK13) infected with a recombinant vaccinia virus LO-R6J20, approximately 2.5×10^4 cells/lane, non-reducing;
lane 4: lysate of a rabbit kidney culture cell line (RK13) infected with the recombinant vaccinia virus LO-R6J20, approximately 2.5×10^4 cells/lane, reducing.

Figure 2 is a calibration curve of a structural protein TrpC11 derived from NANBV as determined by the sandwitch technique using the monoclonal antibodies 5F11 and 5E3 of this invention. Bars in the figure show 2SD (standard deviation).

Figure 3 is a calibration curve of a structural protein TrpC11 derived from NANBV as determined by the sandwitch technique using monoclonal antibodies 1080S and 515S of this invention. Bars show 2SD.

Figure 4 is a calibration curve of a structural protein TrpC11 derived from NANBV as determined by the sandwitch technique using a solid phase monoclonal antibody 5F11 together with β-galactosidase (GAL)-labeled 5E3. Bars show 2SD.

Figure 5 is a calibration curve of a structural protein TrpC11 derived from NANBV as determined by the sandwitch technique using a solid phase monoclonal antibody 1080S together with β-galactosidase (GAL)-labeled 515S. Bars show 2SD.

Figure 6 is a graph showing the detection limit of a structural protein TrpC11 derived from NANBV as determined by the sandwitch technique using the monoclonal antibodies 5F11 and 5E3 of this invention. The concentration of TrpC11 is in the range from 0 to 6,000 pg/ml.

Figure 7 is a graph showing the detection limit of a structural protein TrpC11 derived from NANBV as determined by the sandwitch technique using the monoclonal antibodies 5F11 and 5E3 of this invention. The concentration of TrpC11 is in the range from 0 to 400 pg/ml.

Figure 8 is a graph showing assay results of NANBV structural proteins in serum samples. Samples having a sample number with "N" are ones estimated as negative using a commercially available second generation reagent (Immucheck-HCV Ab "KOKUSAI" sold by International Reagents Corp., Japan), and samples having a sample number with "P" are ones estimated as positive using the same.

We have studied a method for the detection of antigens from the structural protein region of NANBV by use of monoclonal antibodies against them. As a result, we have now found that NANBV virions could be concentrated and dissociated by centrifuging a specimen (e.g., serum) with polyethylene glycol followed by alkaline treatment of the precipitate obtained. Further, we succeeded in obtaining monoclonal antibodies extremely specific for HCV structural proteins using as immunogens recombinant polypeptides having HCV antigenic activity and prepared by expression of genes for core structural proteins in Escherichia coli. The monoclonal antibodies obtained can react specifically with core structural proteins from the viral virion.

Accordingly, this invention provides a monoclonal antibody having a binding specificity for an antigenic determinant site on core structural protein from NANBV.

More specifically, the monoclonal antibody of the invention can be prepared by transforming a host cell with a

obtain a recombinant E. coli.

The culture of the transformed E. coli can be carried out in a usual eutrophic medium for E. coli such as L medium, YT medium or M9-CA medium. The recombinant vector prepared as above has a drug resistance gene, so when the E. coli containing the vector is cultured then it is desirable to add to its medium a corresponding drug of an appropriate concentration. For example, when a recombinant E. coli strain HB101[Trp.TrpE CORE140] that was obtained by transforming E. coli strain HB101 with the recombinant vector Trp.TrpE CORE140 is cultured, ampicillin may be added to the medium in a concentration of 20-200 μg/ml.

The expression of a gene for the fusion polypetide is carried out by inducing the expression by an appropriate promoter upstream of the gene. For example, in the case of the above mentioned vectors, after the transformed host cell is cultured in an appropriate medium until a given level of bacterial cells is obtained, the gene expression can be started by addition of IAA (i.e., indoleacrylic acid). To conduct the efficient gene expression, IAA is preferably added at the early phase or middle phase of the logarithmic growth phase. Following induction of the expression, the culture is continued to accumulate the fusion polypeptide within the bacterial cells. For example, in the case of the recombinant E. coli strain HB101[Trp.TrpE CORE140], it is cultured in an ampicillin-containing M9-CA medium at 37°C for 13-16 hr thereby being obtained in a full amount and producing the fusion polypetide in a high yield.

The collection and purification of fusion polypeptides from cultured bacterial cells can be carried out by conventional techniques, e.g. sonication of the cells, solubilization, ammonium sulfate fractionation, and various chromatographies.

When the fusion polypeptides are expressed efficiently by the above mentioned methods, they form insoluble granules within bacterial cells. In this case the bacterial cells are dispersed in a physiological buffer such as physiological saline and then destructed by for instance sonication, after which the debris is subjected to centrifugation to recover the insoluble materials as precipitate.

The recovered insoluble materials are washed with a buffer containing a low concentration of urea, guanidium HCl or surfactant such as Triton X-100 to obtain a fusion polypeptide having a high purity. The fusion polypetide in the form of insoluble materials can be solubilized by adding thereto a buffer containing 6-8 M urea or guanidium HCl. The solubilized fusion ploypeptide can be dialyzed against or dissolved in an appropriate buffer such as physiological saline so as to decrease the level of urea or guanidium HCl up to an appropriate concentration. The resulting fusion polypetide can be employed as immunogen. If the fusion polypetide is needed to purify in a higher purity, it can be purified using known purification procedures, e.g., separation methods such as salting out, ion-exchange chromatography, gelfiltration and affinity chromatography, fractionation method such as electrophoresis, and combinations thereof.

When a certain base sequence encoding an amino acid sequence that can be cleaved chemically or enzymatically is inserted between the gene fragment encoding NANBV structural protein and the TrpE gene, the fusion polypetide produced is treated by appropriate methods whereby the NANBV antigenic polypeptide that comprises the amino acid sequence shown in SEQ ID NO:1 and is coded for by the gene fragment can be obtained in the TrpE-free form. In this invention, if the part of the amino acid sequence shown in SEQ ID NO:1 includes a substitution, insertion or deletion and the antigenic activity of the polypeptide variant is substantially identical to that of the intact polypetide, such a variant is also included in the scope of the invention.

The term "polypeptide having NANBV antigenic activity" as used herein refers to a polypetide or fusion polypeptide reactive immunologically with an anti-NANBV antibody, and it can be employed as an antigen for use in preparation of hybridomas and monoclonal antibodies. For example, the polypeptide of the invention is a fusion polypeptide having NANBV antigenic activity and comprising the amino acid sequence shown in SEQ ID NO:1, or a polypeptide having NANBV antigenic activity and comprising a part of the amino acid sequence shown in SEQ ID NO:1, wherein to the Nor C-terminus may be added an extra amino acid sequence. As the partial sequence of the amino acid sequence shown in SEQ ID NO:1, preferred are sequences shown in SEQ ID NOs:3, 4 and 5.

Monoclonal antibodies of the invention, directed against the above mentioned fusion polypeptides or polypeptides comprising the amino acid sequence shown in SEQ ID NOs:3, 4 or 5, can be easily prepared. The preparation of a monoclonal antibody by using hybridomas is well known. For example, the following procedures can be employed. BALB/c mice are immunized intraperitonially or subcutaneously at regular intervals of time with each of the above mentioned fusion polypeptides or polypeptides (hereinafter, referred to as "antigen") as a single antigen or as an antigen coupled to BSA, KLH or the like, in combination with complete Freund's adjuvant. At the time when the antibody titer in blood is raised, the mice are boosted with the antigen in the murine tail vein. Following the aseptic removal of spleen cells, they are fused with an appropriate murine myeloma cell line to obtain hybridomas. The preparation method of the monoclonal antibodies of the invention can be carried out by the method of Köhler and Milstein (see Nature 256: 495-497, 1975).

Thus, this invention further provides a hybridoma cell line capable of producing a monoclonal antibody having a binding specificity for an antigenic determinant site on the NANBV core structural protein. More particularly, the hybridoma cell line is selected from the group consisting of HC11-5E3, HC11-5F11, HC11-515S and HC11-1080S. Hybridomas HC11-5E3, HC11-5F11, HC11-515S and HC11-1080S were respectively deposited with the national Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and

pretreatment in which each specimen is treated physically and chemically. That is, polyethylene glycol (PEG) is added to a specimen and then dissolved followed by centrifugation, after which the precipitate is denatured by addition of an alkaline solution thereby the NANBV structural proteins in the specimen being concentrated and disintegrated. By this pretreatment, the high sensitive assay can be achieved. The average molecular weight and content of PEG for use in the treatment of sera can be varied. The average molecular weight of PEG used is typically 1,000, 1,500, 2,000, 4,000 or 6,000, and the content thereof is in the range from 3% to 5% (by weight). PEG having an average molecular weight of 1,000 to 2,000 is advantageously employed in a liquid form after heating because it is a gel and its handling is hard. PEG 4,000 and PEG 6,000 are preferably employed in the pretreatment because they are crystals thus leading to easy handling, and therefore the use of these PEGs is one of preferred embodiments in the invention. Examples of the alkaline agent include, but not limited to, alkali metal or alkaline earth metal hydroxides. The pH of the solution is at least pH 10, preferably pH 12 to 14 during the denaturation treatment.

This invention will be illustrated by the following Examples in more detail, but it should be understood that the invention is not limited only by the Examples.

Example 1

Expression and purification of polypeptide derived from Non-A,Non-B hepatitis virus

(A) Construction of expression plasmid

An expression plasmid that can express a polypeptide of HCV core region was constructed by the method set forth below. One μg of each DNA of plasmids pUC.C11-C21 and pUC.C10-E12 which were obtained by integrating clones C11-C21 or C10-E12 (see JP-A-6-38765) into pUC119, respectively, was digested at 37°C for 1 hr in 20 μl of a restriction reaction solution [50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 100 mM NaCl, 15 units of EcoRI, and 15 units of ClaI] or [10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 50 mM NaCl, 15 units of ClaI, and 15 units of KpnI], respectively. Thereafter, the digests were subjected to 0.8% preparative agarose gel electrophoresis to purify approximately 380 bp EcoRI-ClaI and approximately 920 bp ClaI-KpnI fragments. The two DNA fragments were ligated to a vector that pUC119 had been digested with EcoRI and KpnI at 16°C overnight in 50 μl of a ligase solution which contains a mixture of 5 μl of 10 X ligase buffer [660 mM Tris-HCl (pH 7.5), 66 mM $MgCl_2$, 100 mM dithiothreitol, 1 mM ATP] and 1 μl of T4 ligase (350 units/μl) diluted with water to the total volume of 50 μl. E. coli strain JM109 was transformed with the resultant plasmid to obtain a plasmid pUC.C21-E12.

One ng of the plasmid pUC.C21-E12 DNA was subjected to PCR using two primers: 5'-GAATTCATGGGCAC-GAATCCTAAA-3' (SEQ ID NO:6) and 5'-TTAGTCCTCCAGAACCCGGAC-3' (SEQ ID NO:7). The PCR was carried out using GeneAmp™ Kit (DNA Amplification Reagent Kit, Perkin Elmer Cetus) under the conditions: DNA denaturation, 95°C, 1.5 min; annealing, 50°C, 2 min; and DNA synthesis, 70°C, 3 min.

A DNA fragment obtained by PCR was separated by 0.8% agarose gel electrophoresis and then purified by Glass Powder method (Gene Clean). On the other hand, after pUC19 was digested with SmaI, the above DNA fragment was ligated to the digested pUC19 at 16°C overnight in 50 μl of a ligase solution which contains a mixture of 5 μl of 10 X ligase buffer [660 mM Tris-HCl (pH 7.5), 66 mM $MgCl_2$, 100 mM dithiothreitol, 1 mM ATP] and 1 μl of T4 ligase (350 units/μl) diluted with water to the total volume of 50 μl. E. coli strain JM109 was transformed with the resultant plasmid to obtain a plasmid pUC.19.C21-E12.SmaI. One μg of this plasmid DNA was digested at 37°C for 1 hr in 20 μl of a restriction reaction liquid [150 mM NaCl, 6 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 15 units of EcoRI, and 15 units of BamHI], after which the digest was subjected to 0.8% agarose gel electrophoresis to separate an approximately 490 bp EcoRI-BamHI fragment which was then further purified by Glass Powder method.

Next, 1 μg of an expression vector Trp.TrpE DNA (see JP-A-5-84085) was digested at 37°C for 1 hr in a restriction reaction liquid [150 mM NaCl, 6mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 15 units of EcoRI, and 15 units of BamHI]. To the reaction mixture was added 39 μl of water followed by heat treatment at 70°C for 5 min, after which to this was added 1 μl of bacterial alkaline phosphatase (BAP) (250 units/μl) and the mixture was then left at 37°C for 1 hr. Following addition of phenol, phenol extraction was conducted, the aqueous layer was ethanol-precipitated, and the precipitate was dried. The resultant EcoRI-BamHI treated vector DNA 1 μg was ligated to the above CORE140 fragment at 16°C overnight in 50 μl of a ligase solution which contains a mixture of 5 μl of 10 X ligase buffer [660 mM Tris-HCl (pH 7.5), 66 mM $MgCl_2$, 100 mM dithiothreitol, 1 mM ATP] and 1 μl of T4 ligase (350 units/μl) diluted with water to the total volume of 50 μl.

E. coli strain HB101 was subsequently transformed with 10 μl of the resultant reaction mixture. This competent E. coli strain for use in transformation was prepared by the calcium chloride method (see Mandel, M. and Higa, A. J. Mol. Biol., 53:159-162, 1970). The transformed E. coli was plated onto a LB plate (1% tryptone, 0.5% NaCl and 1.5% agar) containing 25 μg/ml of ampicillin, and cultured at 37°C overnight. A colony on the plate was taken in an amount of 1 platinium loop, which was then transferred to LB medium containing 25 μg/ml of ampicillin followed by culture at 37°C

purified in accordance with usual procedures: precipitation with ammonium sulfate; dialysis against phosphate buffer; and separation of IgG fraction on Protein A-bound Sepharose column. The sub-types of the monoclonal antibodies 5E3, 5F11, 515S and 1080S produced by the above mentioned 4 types of hybridomas were determined as being IgG2a for 5E3 and 5F11 and IgG1 for 515S and 1080S by the double diffusion method using each of the isotypes of rabbit anti-mouse Ig (Zymed). Regarding 4 types of the monoclonal antibodies, epitope analyses were conducted using 20 peptides synthesized based on sequences from the HCV core region, and as a result, it has been found that they could recognize parts of the core region specifically. The results are shown in Table 1.

Table 1

Antibody	Recognition site
5E3	^{25}Pro - ^{35}Tyr (SEQ ID NO:3)
5F11	^{51}Lys - ^{60}Gly (SEQ ID NO:5)
515S	^{25}Pro - ^{35}Tyr (SEQ ID NO:3)
1080S	^{30}Ile - ^{50}Arg (SEQ ID NO:4)

Example 4

Demonstration of reaction specificity of the antibodies by Western blotting

Lysates containing proteins expressed within a culture cell line derived from rabbit kidney, RK13, that has been separately infected with a recombinant vaccinia virus LO-R6J20 which carries a gene encoding CORE, ENV, E2/NS1 and NS2 regions from HCV or with a recombinant vaccinia virus LO-R6J13 which carries a gene encoding ENV and E2/NS1 regions from HCV, were subjected to SDS-polyacrylamide gel electrophoresis, after which the resultant gel was attached closely to a polyvinylidene fluoride membrane (PVDF membrane, Millipore) and electroblotted (gel side: cathode; PVDF membrane side: anode) to transfer the proteins on the gel to the PVDF membrane Then the transferred PVDF membrane was soaked at 4°C overnight in 0.1 M phosphate buffer (pH 7.4) containing 5% skim milk (Difco) and 1% BSA (bovine serum albumin) in order to effect blocking and washed with Tris-HCl buffer (pH 7.0) containing 0.05% Tween 20 (referred to as "TBS").

The PVDF was subsequently reacted at room temperature for 1 hr with 10 μg/ml of a monoclonal antibody chosen from 515S, 1080S, 5E3 and 5F11, as first antibody, prepared in Example 3 and dissolved in 0.1 M phosphate buffer (pH 7.4) containing 5% skim milk and 1% BSA. After reaction, the PVDF membrane was washed well with TBS and then reacted at room temperature for additional 1 hr with a horse radish peroxidase-labeled anti-mouse IgG+IgM antibody mixture, as second antibody, diluted in 0.1 M phosphate buffer (pH 7.4) containing 5% skim milk and 1% BSA. After the resultant PVDF membrane was washed well with TBS, the color development was done using 0.1% 4-chloro-1-naphtol solution and 0.2% H_2O_2 solution.

Western blot images between each monoclonal antibody and HCV.CORE antigen are shown in Fig. 1. Each of the monoclonal antibodies was specifically reacted with a 22 Kd protein expressed in the recombinant vaccinia virus-infected eukaryote cells that contain an HCV.CORE region encoding gene, but not with proteins expressed in the recombinant vaccinia virus-infected eukaryote cells that contain a gene encoding HCV.ENV and E2/NS1 regions. Thus these results prove that the monoclonal antibodies employed can recognize an HCV.CORE antigen specifically.

Example 5

Preparation of β-galactosidase labeled monoclonal antibody

(A) Preparation of 5E3F(ab')$_2$

To use an anti-HCV.CORE antibody (i.e., mouse monoclonal antibody: 5E3IgG) for labeling, 10 mg of the antibody was dialyzed against 0.1 M sodium acetate buffer (pH 4.0) containing 0.2 M NaCl, and then concentrated to a volume

addition of 100 µl of 0.5 M NaH_2PO_4 containing 5% Triton X-100.

Example 8

Detection and quantitative analysis of Non-A,Non-B hepatitis structural region core proteins by use of the monoclonal antibodies 5F11 and 5E3

According to the method described in Example 6, the monoclonal antibody 5F11 was immobilized on a microplate to which Blocking buffer was then added and left at 4°C overnight, thereby a 5F11 immobilized support being prepared. To each well of the plate were added 100 µl of 10 mM sodium phosphate buffer (pH 7.0) containing 0.5 M NaCl, 2.5 mM EDTA.2Na, 1% BSA, 0.5% casein-Na, 5% mouse serum and 0.25% Tween 80 (hereinafter, referred to as "Reaction buffer") and 50 µl of each of the solutions of TrpC11 protein having different concentrations ranging from 0 to 100 ng/ml prepared in Example 1, and the plate was reacted at room temperature for 1 hr while shaking and washed six times with 300 µl each of Washing buffer. The plate was then reacted at room temperature for 1 hr following the addition of 100 µl of the peroxydase (POD) labeled monoclonal antibody 5E3.

After the plate was washed six times with 300 µl of Washing buffer, to which 100 µl of a solution of the substrate o-phenylene diamine (hereinafter, referred to as "OPD") was added, and the plate was reacted at room temperature for 30 min. To the plate, 100 µl of a reaction termination liquid of 2N sulfuric acid was added, after which the amount of TrpC11 protein was determined by measurement of the absorbance (A_{492}) at 492 nm in relative to the absorbance at 690 nm as control. Table 2 shows that the amount of TrpC11 protein can be measured in a dose dependent manner. Thus, by use of the monoclonal antibodies 5F11 and 5E3 of the invention, NANBV structural region core proteins can be detected or determined quantitatively.

Example 9

Detection and quantitative analysis of Non-A,Non-B hepatitis structural region core proteins by use of the monoclonal antibodies 1080S and 515S

The TrpC11 protein was measured by repeating the procedure of Example 8, except that the monclonal antibody 1080S and the monoclonal antibody 515S were employed as a solid phase antibody and as a labeled antibody, respectively. As shown in Fig. 3, it is found that the TrpC11 protein can be measured in a dose dependent manner. This result proves that the use of the monoclonal antibodies 1080S and 515S make it possible to detect or quantitatively determine NANBV structural region core proteins.

Example 10

Comparison of sensitivity for measurement of Non-A,Non-B hepatitis virus structural region core proteins by means of different enzyme-labeled antibodies

The measurement of the TrpC11 protein was substantially carried out in the same way as in Example 8, except that POD, alkaline phosphatase or β-galactosidase was employed as an enzyme for labeling of second antibodies and that OPD (o-phenylene diamine), HPPA (3-(4-hydroxyphenyl)propionic acid), pNPP (p-nitrophenyl phosphate), NADP (nicotineamide adenine dinucleotide phosphate), AMPPD (3-(2'-spiroadamantane)-4-methoxy-4-(3"-phosphoryloxy)-phenyl-1,2-dioxetane) or 4MUG (4-methylumbelliferyl β-D-galactopyranoside) was employed as a substrate. When a combination of the monoclonal antibodies 1080S and 515S was employed, as seen in Table 2, the system of β-galactosidase/4MUG gave the highest sensitivity for detection of NANBV structural region core proteins. And the high sensitivity was achieved when the monoclonal antibody 5F11 was employed as a solid phase antibody while the β-galactosidase-labeled monoclonal antibody 5E3 as a second aontibody (see Figs. 4 and 5).

Table 2

		Detection limit	
Enzyme	Substrate	Enzyme	TrpC11
POD	OPD (colorimetry)	30 amol/assay	1,560 pg/ml
POD	HPPA (fluorometry)	15	1,000
ALP	pNPP (colorimetry)	30	20,000
ALP	NADP (colorimetry)	10	1,000

Example 13

Measurement of reaction rate constants of the monoclonal antibodies 5F11, 5E3, 1080S and 515S

Rate constants of the immunological reaction between each of the monoclonal antibodies 5F11, 5E3, 1080S and 515S and TrpC11 protein were determined using a surface plasmon reasonance analyzer (BIAcore™, Pharmacia) that was an instrument for measurement of biospecific interactions based upon the principle of surface plasmon resonance. The TrpC11 protein was immobilized onto Sensortip™ CM5 (Pharmacia) having a carboxydextran layer by the usual amine coupling method, and each of the monoclonal antibodies that were diluted to an appropriate concentration in HBS buffer (10mM HEPES, 3.4 mM EDTA, 150 mM NaCl, 0.005% Tween 20, pH 7.4) was flowed through the Sensortip over 25 min. In this case, the resonance signal value that increases by binding of each antibody to the TrpC11 protein on Sensortrip, was measured at time intervals of 30 sec. At the same time, the change in resonance signal was calculated and recorded. The samples were prepared in 5 to 10 different concentrations per antibody and measured in the same way as above to determine an affinity rate constant.

On the other hand, for the measurement of a dissociation rate constant, 100 μg/ml monoclonal antibody in HBS buffer was flowed through the TrpC11 immobilized Sensortip over 15 min to bind the antibody to the TrpC11 on Sensortip. HBS buffer was then passed through the Sensortip over 50 min, and the change in resonance signal resulted from the dissociation of the antibody from the TrpC11 on Sensortip was measured at time intervals of 60 sec. From the ratio of the affinity rate constant and dissociation rate constant determined in the same way as above, the binding constant was calculated. The results are shown in Table 3.

Table 3

Antibody	Affinity rate constant (K_{+1} ($M^{-1}s^{-1}$)	Dissociation rate constant k_{-1} (s^{-1})	Binding constant K_A (M^{-1})
5E3	2.2×10^5	$< 10^{-5}$	$> 2.2 \times 10^{10}$
5F11	4.1×10^4	5.7×10^{-5}	7.2×10^8
515S	8.1×10^4	$< 10^{-5}$	$> 8.1 \times 10^9$
1080S	1.9×10^5	2.3×10^{-4}	8.3×10^8

From the table, all the tested monoclonal antibodies have a binding constant of not less than 10^8 (M^{-1}), indicating that they have high affinity for the target antigen.

Advantages of the Invention

According to this invention, hybridoma cell lines that can produce a monoclonal antibody having high binding specificity for an antigenic determinant on NANBV core structural proteins were obtained. The valuable monoclonal antibodies can specifically recognize the NANBV structural proteins in sera from patients with Non-A,Non-B hepatitis thereby being served extensively as antibodies in various immunological reagents for diagnosis of NonA,Non-B hepatitis. Additionally, the definitive diagnosis of NonA,Non-B hepatitis can be conducted by utilizing the detection or quantification method of NANBV with the monoclonal antibodies of the invention.

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(A) TELEPHONE:

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 140 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vi) ORIGINAL SOURCE:

(A) ORGANISM: NON-A,NON-B hepatitis virus

(xi) SEQUENCING DESCRIPTION: SEQ ID NO:1:

Met Lys Ala Ile Phe Val Leu Lys Gly Ser Leu Asp Arg Asp Pro Glu
1 5 10 15

Phe Met Gly Thr Asn Pro Lys Pro Gln Arg Lys Thr Lys Arg Asn Thr
20 25 30

Asn Arg Arg Pro Gln Asp Val Lys Phe Pro Gly Gly Gly Gln Ile Val
35 40 45

Thr Arg Lys Thr Ser Lys Arg Ser Gln Pro Arg Gly Gly Arg Arg Pro
50 55 60

Ile Pro Lys Asp Arg Arg Ser Thr Gly Lys Ser Trp Gly Lys Pro Gly
65 70 75 80

Tyr Pro Trp pro Leu Tyr Gly Asn Glu Gly Leu Gly Trp Ala Gly Trp
85 90 95

Leu Leu Ser Pro Arg Gly Ser Arg Pro Ser Trp Gly Pro Thr Asp Pro
100 105 110

Arg His Arg Ser Arg Asn Val Gly Lys Val Ile
115 120

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 11 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vi) ORIGINAL SOURCE:

(A) ORGANISM: NON-A,NON-B hepatitis virus

(xi) SEQUENCING DESCRIPTION: SEQ ID NO:3:

Pro Gly Gly Gly Gln Ile Val Gly Gly Val Tyr
1 5 10

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: linear

GAATTCATGG GCACGAATCC TAAA 24

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCING DESCRIPTION: SEQ ID NO:7:

TTAGTCCTCC AGAACCCGGA C 21

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 140 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vi) ORIGINAL SOURCE:
(A) ORGANISM: NON-A,NON-B hepatitis virus

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
Met Lys Ala Ile Phe Val Leu Lys Gly Ser Leu Asp Arg Asp Pro Glu
 1               5                  10                  15
Phe Met Gly Thr Asn Pro Lys Pro Gln Arg Lys Thr Lys Arg Asn Thr
            20                  25                  30
Asn Arg Arg Pro Gln Asp Val Lys Phe Pro Gly Gly Gly Gln Ile Val
        35                  40                  45
Gly Gly Val Tyr Leu Leu Pro Arg Arg Gly Pro Arg Leu Gly Val Arg
    50                  55                  60
Ala Thr Arg Lys Thr Ser Lys Arg Ser Gln Pro Arg Gly Gly Arg Arg
65                  70                  75                  80
Pro Ile Pro Lys Asp Arg Arg Ser Thr Gly Lys Ser Trp Gly Lys Pro
                85                  90                  95
Gly Tyr Pro Trp Pro Leu Tyr Gly Asn Glu Gly Leu Gly Trp Ala Gly
            100                 105                 110
Trp Leu Leu Ser Pro Arg Gly Ser Arg Pro Ser Trp Gly Pro Thr Asp
        115                 120                 125
Pro Arg His Arg Ser Arg Asn Val Gly Lys Val Ile
    130                 135                 140
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 123 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vi) ORIGINAL SOURCE:
(A) ORGANISM: NON-A,NON-B hepatitis virus

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vi) ORIGINAL SOURCE:

(A) ORGANISM: NON-A,NON-B hepatitis virus

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Lys Thr Ser Lys Arg Ser Gln Pro Arg Gly
1 5 10

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid: synthetic primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GAATTCATGG GCACGAATCC TAAA 24

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid: synthetic primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TTAGTCCTCC AGAACCCGGA C 21

Claims

1. A hybridoma cell line which can produce a monoclonal antibody having binding specificity for an antigenic determinant site on core structural protein from Non-A,Non-B hepatitis virus (NANBV), the monoclonal antibody having a binding constant K_A of 5×10^7 [M^{-1}] or larger when forming an immune complex between the monoclonal antibody and the NANBV core structural protein.

2. The hybridoma cell line of claim 1 which is selected from the group consisting of HC11-5E3 (FERM BP-5147), HC11-5F11 (FERM BP-5148), HC11-515S (FERM BP-5146) and HC11-1080S (FERM BP-5145).

3. A monoclonal antibody produced by the hybridoma cell line according to claim 1 or claim 2.

antibody defined in any of claims 3 to 8, wherein these monoclonal antibodies may be identical or different.

19. The test kit of claim 18 wherein the monoclonal antibody is selected from the group consisting of 5E3, 5F11, 515S and 1080S which have binding specificity for an antigenic determinant site on NANBV core structural protein, and the labeled second antibody is selected from the group consisting of labeled 5E3, 5F11, 515S and 1080S.

20. A test kit for use in the immunoassay according to claim 16 or claim 17, which comprises at least one monoclonal antibody defined in any of claims 3 to 8.

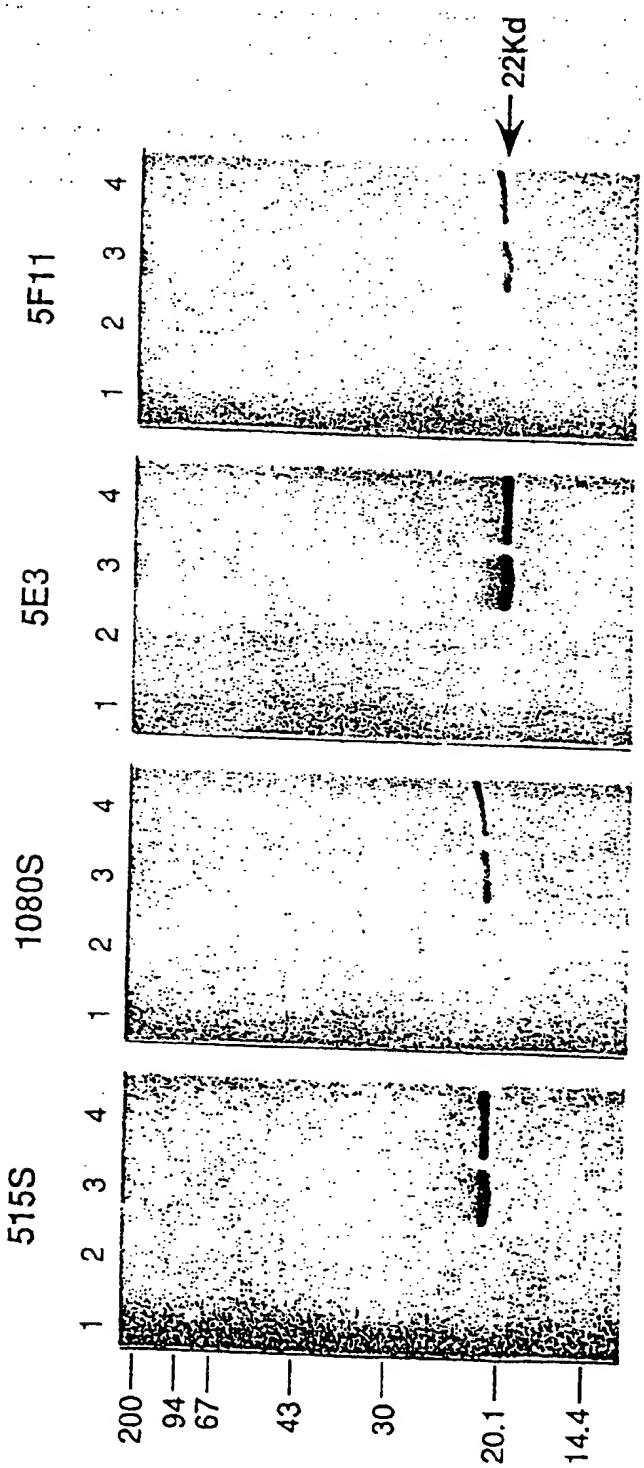


Fig. 1

Fig. 2

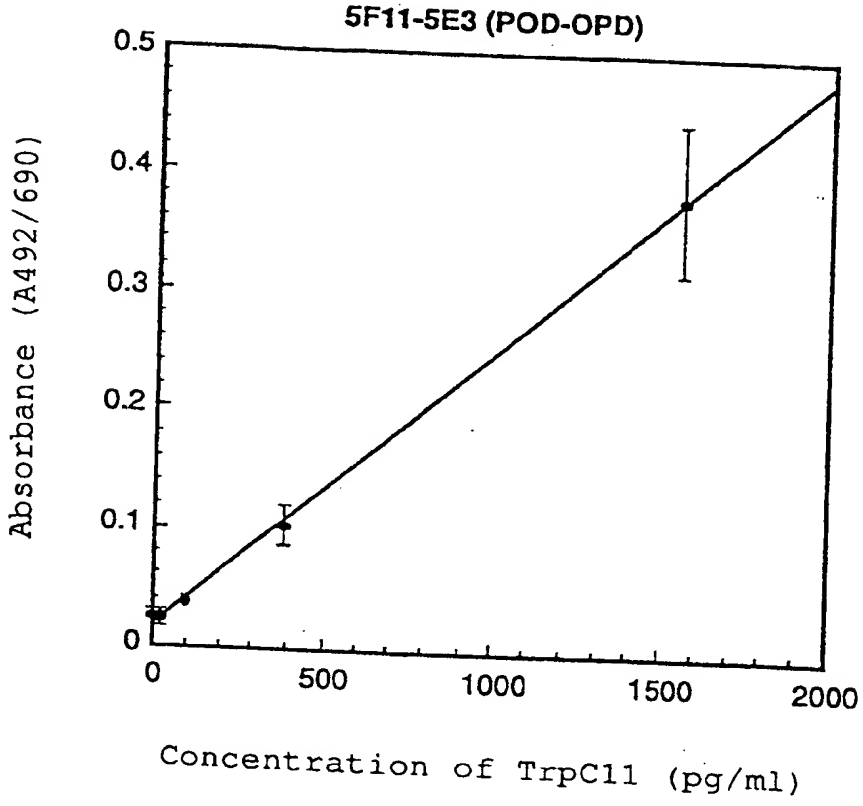

5F11-5E3 (POD-OPD)
Absorbance (A492/690)
0.5
0.4
0.3
0.2
0.1
0
0
500
1000
1500
2000
Concentration of TrpC11 (pg/ml)

Fig. 3

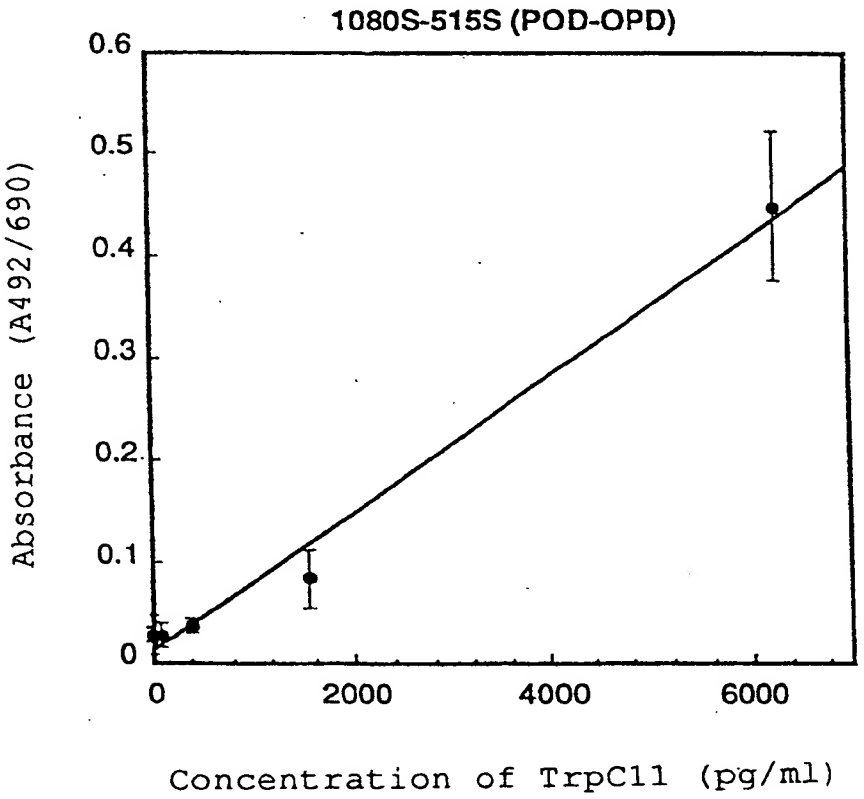

1080S-515S (POD-OPD)
Absorbance (A492/690)
0.6
0.5
0.4
0.3
0.2
0.1
0
0
2000
4000
6000
Concentration of TrpC11 (pg/ml)

Fig. 4

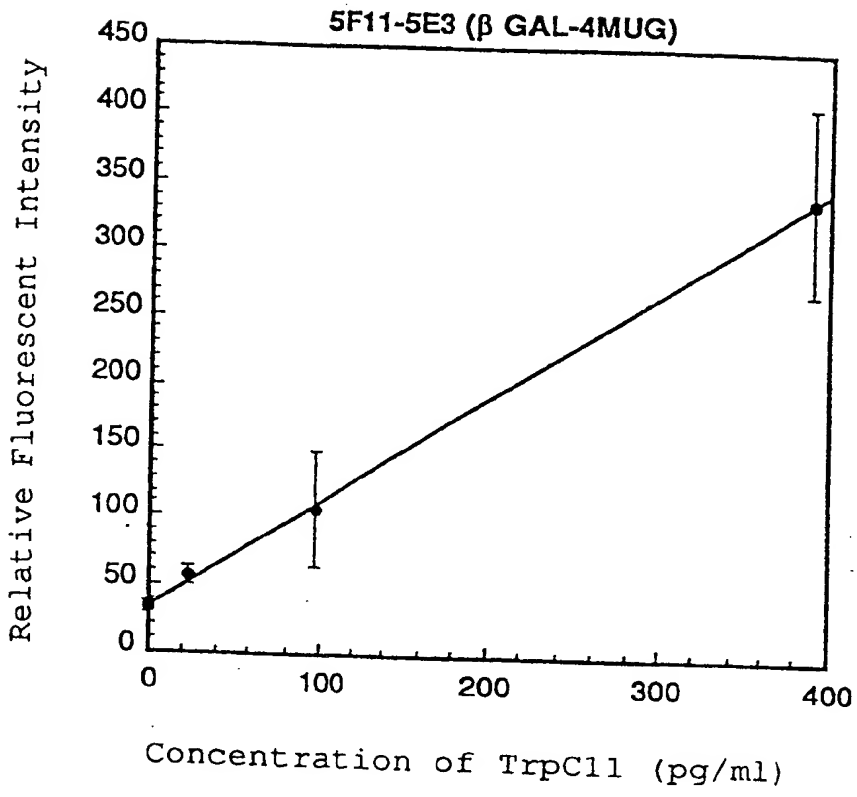

5F11-5E3 (β GAL-4MUG)
Relative Fluorescent Intensity
450
400
350
300
250
200
150
100
50
0
0
100
200
300
400
Concentration of TrpC11 (pg/ml)

Fig. 5

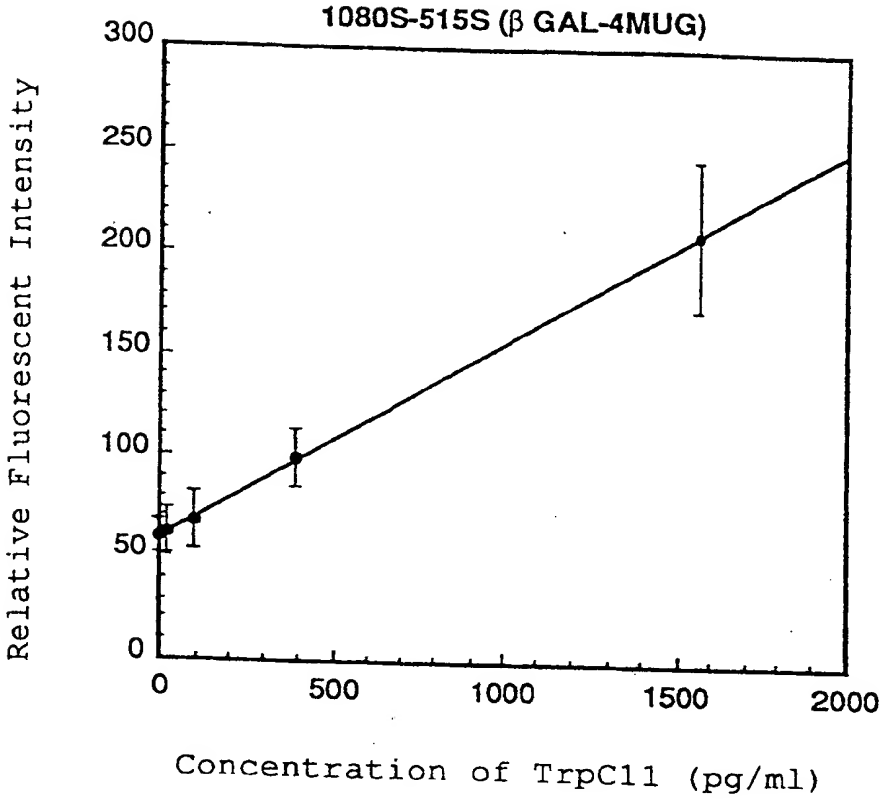

1080S-515S (β GAL-4MUG)
Relative Fluorescent Intensity
300
250
200
150
100
50
0
0
500
1000
1500
2000
Concentration of TrpC11 (pg/ml)

Fig. 6

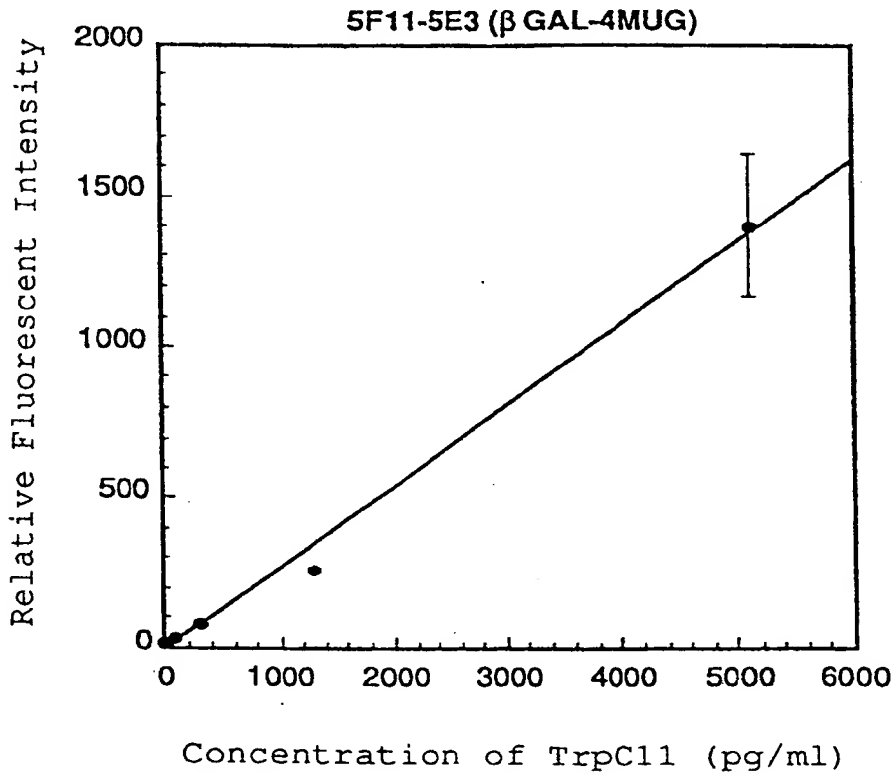

5F11-5E3 (β GAL-4MUG)
Relative Fluorescent Intensity
2000
1500
1000
500
0
0
1000
2000
3000
4000
5000
6000
Concentration of TrpC11 (pg/ml)

Fig. 7

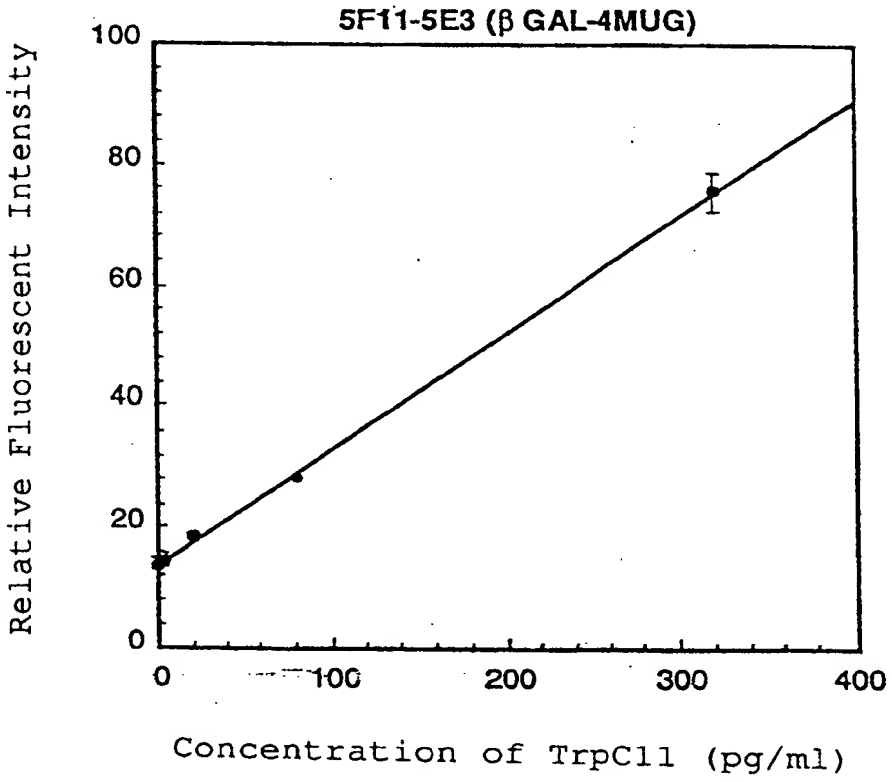
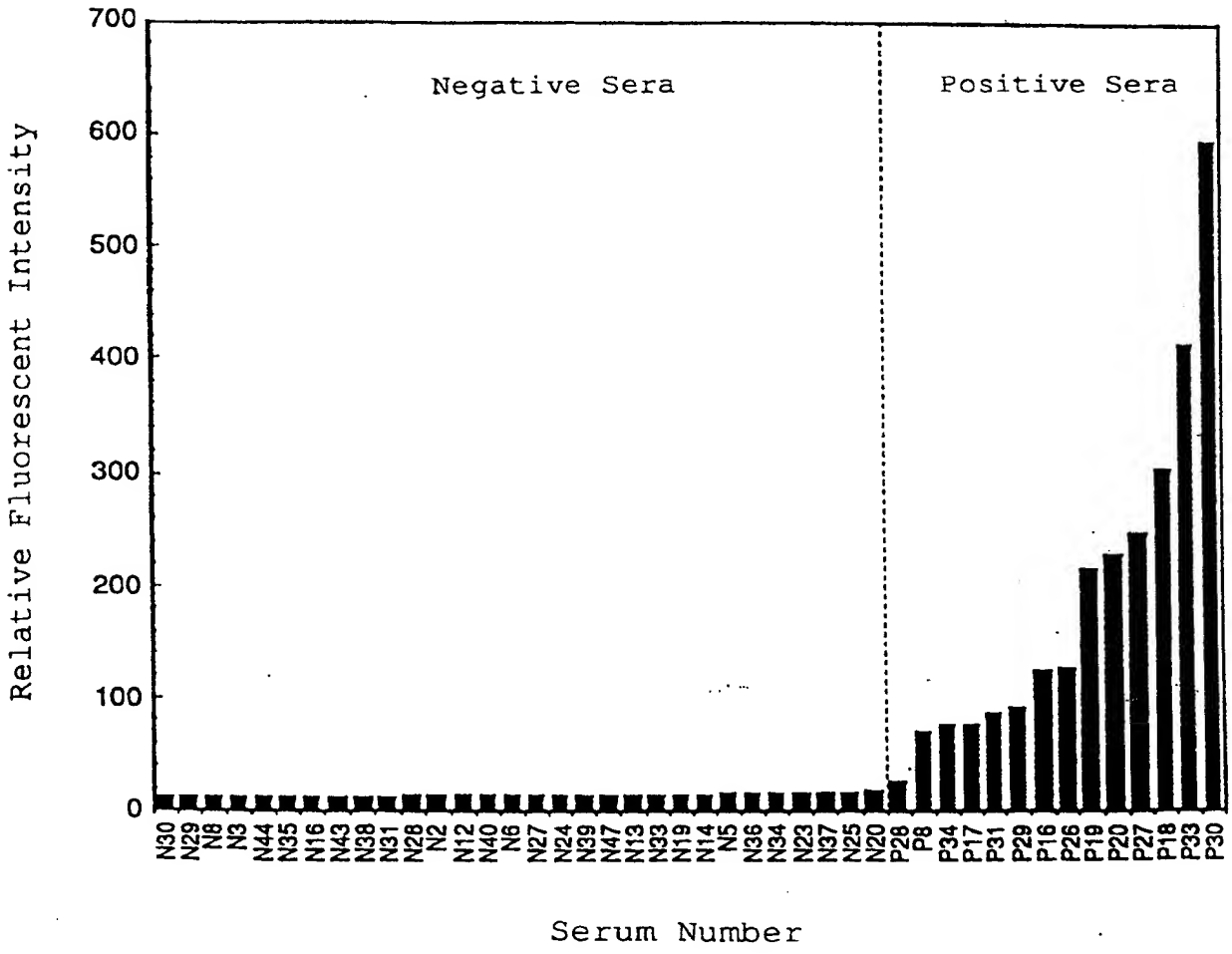

5F11-5E3 (β GAL-4MUG)
Relative Fluorescent Intensity
100
80
60
40
20
0
0
100
200
300
400
Concentration of TrpC11 (pg/ml)

Fig. 8


Negative Sera
Positive Sera
Relative Fluorescent Intensity
700
600
500
400
300
200
100
0
N30
N29
N8
N3
N44
N35
N16
N43
N38
N31
N28
N2
N12
N40
N6
N27
N24
N39
N47
N13
N33
N19
N14
N5
N36
N34
N23
N37
N25
N20
P28
P8
P34
P17
P31
P29
P16
P26
P19
P20
P27
P18
P33
P30
Serum Number